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Isolation of Myelin Basic Protein-Reactive T-Cell Lines from Normal Human Blood

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T-Cell lines which responded by proliferation to the autoantigen, myelin basic protein (MBP), were isolated from the blood of six of nine normal humans. These T-cell lines could be maintained in *in vitro* culture for up to 2 months through the use of Interleukin 2 and repeated MBP stimulation. Optimal antigen-induced proliferation required both antigen and antigen-presenting cells found in the adherent cell population of autologous peripheral blood mononuclear cells (PBM). The T-cell lines were predominantly of the helper phenotype (OKT3$^+$, OKT4$^+$, OKT8$^-$) and responded to both human and guinea pig myelin basic protein.

INTRODUCTION

Experimental allergic encephalomyelitis (EAE)[3] is often proposed as an animal model for multiple sclerosis (MS) due to the histologic and clinical similarities shared by these disorders (1–5). This experimental disease may be induced in animals by sensitization to myelin basic protein (MBP) with the subsequent appearance of an easily detected cellular immunity to this antigen (6, 7). For this reason, many investigators have attempted to determine whether or not the peripheral blood mononuclear cells (PBM) from individuals with MS display an increased cellular immune response to myelin basic protein compared to PBM from control donors. Different assay methods have been used with often contradictory results (reviewed in Refs. (8, 9)). In two recent studies of lymphocyte transformation induced by MBP, the most consistent finding has been a general correlation between slightly higher levels of proliferation induced by MBP and increasing duration of disease (10, 11). However, in these studies the level of responsiveness to MBP has been very modest with considerable overlap between the responses of PBM from MS and control subjects. For this reason, Hughes *et al.* suggested that a low level of sensitization to MBP might

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[3] Abbreviations used: IL-2, Interleukin 2; EAE, experimental allergic encephalomyelitis; MS, multiple sclerosis; MBP, myelin basic protein; PBM, peripheral blood mononuclear cells; TT, tetanus toxoid; CTC, cultured T cells.

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be present even in normal subjects (10). However, the techniques used in these studies did not permit the isolation of sufficient numbers of cells to confirm the specificity of the response to MBP or to characterize the phenotype of the responding lymphocytes.

We now report the preliminary characterization of MBP-reactive T-cell lines isolated from normal human blood. Primary *in vitro* sensitization to MBP was employed with long-term *in vitro* culture of responding lymphocytes in T-cell growth factor (also called Interleukin 2; IL-2) (12–17). Normal subjects were studied prior to extending these investigations to individuals with MS.

MATERIALS AND METHODS

Subjects. Two men and seven women ages 25–35 years, with no history of neurologic disease, were studied. Two individuals had possible prior exposure to myelin basic protein during laboratory work, and three others were involved in virology research. The four other subjects had no history of possible exogenous exposure to CNS antigens.

Lymphocyte and adherent cell populations. Peripheral blood mononuclear cells were isolated from normal donors by Ficoll–Hypaque gradients (Pharmacia Fine Chemicals, Piscataway, N.J.) in the standard manner and washed three times (18). For isolation of adherent cells, the method of Kumagai *et al.* was followed (19). The average yield of adherent cells was 4 to 5% of the initial PBM sample. In prior studies, less than 1% of the adherent cells prepared in this manner bore the T-cell markers OKT3 or OKT4, while 93% bore the OKM1 marker thought to predominantly label monocytes and macrophages (20, 21).

Antigens. Guinea pig MBP (GP-MBP) and human MBP (H-MBP) were prepared by the method of Diebler *et al.* (22) and provided by Dr. David Pleasure. A second preparation of GP-MBP, generously provided by Dr. Marian Kies, was also used in selected experiments to confirm specificity of the response. Partially purified tetanus toxoid (TT) (Lot LP445 PR) was purchased from the Commonwealth of Massachusetts, Department of Public Health, Boston, Massachusetts.

Isolation of antigen-specific cell lines. PBM from normal donors was cultured in supplemented RPMI 1640 (GIBCO, Grand Island, N.Y.) (supplemented with antibiotics, 2 m*M* glutamine, 1% nonessential amino acids (GIBCO), and 1 m*M* sodium pyruvate (GIBCO)) containing 10% autologous serum at 2×10^6 cell/ml. Either GP-MBP (40 μg/ml) (prepared by Dr. Pleasure) or TT (0.5 lf units/ml) was added to 2.5-ml cultures for a 6- to 7-day incubation at 37°C in 5% CO_2/air. Following the above culture, supplemented RPMI containing 10% fetal calf serum (FCS, Microbiological Associates, Walkersville, Md.) and 10% mitogen-depleted IL-2-containing medium, prepared as described previously, was added (21). The cultures were examined daily by inverted microscopy and were divided and refed as necessary with the RPMI medium supplemented with FCS and IL-2. When proliferation under these conditions slowed, the cultured T cells (CTC) were washed and 2×10^5 CTC were placed in 2.5 ml supplemented RPMI containing (1) 10% autologous serum; (2) 5×10^6 autologous irradiated (2000 R) PBM; and (3) an optimal concentration of tetanus toxoid or GP-MBP. Cultures were continued for 3 days and responding cells were placed again in culture with IL-2-supplemented medium until it was necessary to repeat the above procedure. Using this approach, proliferating GP-MBP-reactive T cells were maintained in continuous culture for up to 2 months.

Antigen specificity assay of cultured T cells. T-Cell lines were examined for antigen-specific proliferation in a 48-hr assay by measurement of incorporation of tritiated thymidine ([^{3}H]Tdr; New England Nuclear, Boston, Mass.). Before use in these antigen-specific proliferation assays, cultures of T cells were maintained in IL-2 alone, without additional feeder cells or antigen for 3 to 5 days. Fifteen thousand cultured T cells were incubated in 0.2 ml of supplemented RPMI with 10% autologous serum, 1×10^5 irradiated fresh PBM, or 2×10^4 irradiated adherent cells, and antigen or medium alone. [^{3}H]Tdr, 1 μCi/well was added for the final 4 hr of culture and isotope incorporation measured by liquid scintillation spectroscopy.

Surface marker phenotype of cultured T cells. Surface marker characteristics of cultured T cells were determined as previously described by indirect immunofluorescence for T-cell subsets (21, 23). In these studies, aliquots of the cultured cells were washed with chilled medium and incubated in cold solutions containing one of a set of monoclonal antibodies; OKT3, OKT4, or OKT8 (Ortho Pharmaceutical Co., Raritan, N.J.) (20, 23). The cells were then washed and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Cappel, Cochranville, Pa.). Between 100 and 200 cells were counted.

RESULTS

Myelin Basic Protein-Reactive Lymphocytes

Lymphocytes which responded *in vitro* to myelin basic protein were recovered from six of nine subjects studied. GP-MBP-reactive lymphocytes were recovered from three of the four subjects having no known exogenous exposure to CNS antigens and from three of the five remaining individuals who could have been exposed to CNS antigens while performing laboratory duties. GP-MBP-reactive cells were isolated successfully in three separate experiments from subject No. 1 and in two separate experiments from subject No. 3 (data not shown). For each of the subjects studied, two separate T-cell lines, one responding to GP-MBP and the other to TT, were established and studied in parallel for determination of the specificity of antigen response. Table 1 shows antigen specificity studies using T cells from subject No. 1, recognizing either TT or GP-MBP. T cells selected for response to TT did not respond to GP-MBP and those selected for response to GP-MBP did not proliferate in response to TT. Autologous, irradiated whole PBM or adherent cells were necessary as a source of antigen-presenting cells for optimal response to both TT and GP-MBP.

The GP-MBP preparation used for *in vitro* sensitization was encephalitogenic for Lewis rats. However, to lessen the possibility that the CTC were responding to an antigen other than MBP, which might have been present in our preparation, a second source of GP-MBP, kindly provided by Dr. Marian Kies, was also employed as an antigen. In addition, since there are minor differences in amino acid composition between human and guinea pig myelin basic protein (24), the response of GP-MBP-reactive T cells to human MBP was also tested. Table 2 shows the results of these studies using the three different preparations of myelin basic protein and CTC from two different individuals. In each assay, the MBP-reactive CTC of each individual responded to each preparation of MBP, but not to tetanus toxoid. Each of three MBP-reactive T-cell lines isolated from different donors is predominantly OKT3 positive, OKT4 positive, and OKT8 negative (Table 3).

TABLE 1

Antigen Specificity of Human GP-MBP-Reactive T Cells (Subject No. 1)[a]

Culture condition	MBP-CTC	TT-CTC
1. Medium alone	105 ± 29	533 ± 175
2. GP-MBP	1,915 ± 302	ND[b]
3. TT	128 ± 8	183 ± 11
4. PBM[c] alone	497 ± 192	2,289 ± 186
5. Adh[d] alone	843 ± 152	2,397 ± 318
6. PBM + GP-MBP	49,238 ± 1867	ND
7. Adh + GP-MBP	69,297 ± 3267	2,159 ± 438
8. Adh + TT	766 ± 75	26,946 ± 2383
9. PBM + TT	ND	21,253 ± 1572

[a] T-Cell lines responding to either GP-MBP (MBP-CTC) or tetanus toxoid (TT-CTC) were isolated as described from normal subject No. 1. Fifteen thousand T cells were cultured with (1) antigen alone (GP-MBP or TT); (2) antigen-presenting cells alone (autologous irradiated PBM (1×10^5/microwell) or irradiated adherent cells (2×10^4/microwell)); or (3) antigen plus antigen-presenting cells. Stimulation was determined by incorporation of [^{3}H]TdR. Values represent mean counts per minute (cpm) ± SEM of triplicate cultures.

[b] Not determined.

[c] PBM, irradiated (2000 R) peripheral blood mononuclear cells (1×10^5/microwell).

[d] Adh, irradiated adherent cells (2×10^4/microwell).

DISCUSSION

The major finding reported here is that helper phenotype T cells responsive to MBP are present in the blood of normal humans. None of the subjects studied had any history of neurologic disease. These MBP-reactive T cells can be isolated and expanded in number in cultures with added IL-2. The antigen-reactive T cells were almost exclusively of helper phenotype and proliferated in response to both guinea

TABLE 2

Proliferative Response to Human Myelin Basic Protein[a]

Culture conditions	MBP-CTC	
	Subject No. 1	Subject No. 2
1. Medium alone	283 ± 51	205 ± 20
2. PBM[b]	2,267 ± 594	383 ± 15
3. PBM + TT	1,391 ± 261	372 ± 67
4. PBM + No. 1 GP-MBP[c]	22,165 ± 884	22,258 ± 614
5. PBM + No. 2 GP-MBP[d]	23,447 ± 1029	24,423 ± 493
6. PBM + H-MBP[e]	17,906 ± 1853	15,655 ± 351

[a] T-Cell lines which responded to GP-MBP (MBP-CTC) were isolated, as described, from normal subjects No. 1 and No. 2. Antigen-induced proliferation was determined by culture of 1.5×10^4 CTC with autologous irradiated PBM and either tetanus toxoid (TT) or MBP (three different preparations were studied). Stimulation was assessed by incorporation of [^{3}H]TdR. Values represent mean cpm ± SEM of triplicate cultures.

[b] PBM, irradiated autologous peripheral blood mononuclear cells (1×10^5/microwell).

[c] No. 1 GP-MBP, guinea pig myelin basic protein provided by Dr. Marian Kies.

[d] No. 2 GP-MBP, guinea pig myelin basic protein provided by Dr. David Pleasure.

[e] Human-MBP, human myelin basic protein provided by Dr. David Pleasure.

TABLE 3

OKT Phenotype of Human MBP-Reactive Lymphocytes[a]

	Percentage		
	OKT3	OKT4	OKT8
No. 1 MBP-CTC	86	100	0
No. 2 MBP-CTC	81	66	1
No. 3 MBP-CTC	100	92	1

[a] T-Cell lines responding to guinea pig myelin basic protein (MBP-CTC) were isolated from three normal subjects. Cells (1×10^6) were stained with mouse anti-T-cell subset hybridoma antibodies and fluorescein-conjugated anti-mouse IgG. Between 100 and 200 cells were examined.

pig myelin basic protein and human myelin basic protein. Optimal proliferation required antigen-presenting cells, which are present in the adherent cell population of autologous PBM. Both the requirement for antigen-presenting cells and the predominantly helper phenotype have been previously reported to characterize human T cells reactive with standard soluble antigens (15–17).

The biologic significance of such MBP-reactive helper phenotype T cells in normal blood is uncertain. Animal studies have shown that the lymphocyte subset responsible for adoptive transfer of EAE is the helper-T-cell subset which also proliferates when cultured *in vitro* with myelin basic protein (6, 25). Adoptive transfer of EAE can be effected with much smaller numbers of such MBP-reactive cells than freshly obtained lymphocytes from animals immunized with myelin basic protein (26, 27). Whether or not human helper phenotype T cells recognizing MBP or some other CNS antigen play any role in the initial or subsequent episodes of demyelination in MS is unknown. However, indirect evidence for this is provided by the recent study of the distribution of T-cell subsets in active chronic MS lesions (28). Traugott *et al.* presented data suggesting that helper phenotype T cells are actively involved in extension of the demyelinated lesion of MS. The present study provides the first demonstration that there are helper phenotype T cells in the PBM of normal individuals which recognize an intrinsic, and sometimes encephalitogenic, protein constituent of normal myelin.

The immune mechanisms responsible for induction and maintenance of tolerance to self constituents in humans are not well understood. It has been considered that autoimmune reactivity leading to disease does not occur under normal circumstances because immune cells recognizing self antigens are either deleted during development or may be under active immunoregulatory control (reviewed in Ref. (29)). The findings presented in this report argue more for the active immunoregulation of the response to MBP since T cells reactive with this antigen can be recovered from some normal individuals. Fluctuations in the level of circulating T-cell subsets occur during MS exacerbations, with a diminished level of suppressor cells noted at the onset of an exacerbation (30, 31). It is possible that this generalized depression of suppressor activity is associated with a local defect in immunoregulation which permits an autoimmune response to occur within the central nervous system. In animal studies, there is evidence that suppressor T cells are important in establishing and maintaining resistance to EAE, possibly through specific suppression of the MBP-reactive T-cell population (32–34). Lamb and Feldman have recently reported the isolation of a

human suppressor T-cell clone which recognizes an autologous helper-T-cell clone reactive with a protein component of the influenza virus (35). The use of human T-cell lines reactive with myelin basic protein may permit similar delineation of immune mechanisms which may control this autoantigen-reactive cell population in both normal subjects and MS patients.

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REFERENCES

1. Paterson, P. Y., *In* "Autoimmunity" (N. Talal, Ed.), pp. 644–692. Academic Press, New York, 1977.
2. Rostami, A., Lisak, R. P., Blanchard, N., Guerrero, F., Zweiman, B., and Pleasure, D. E., *J. Neurol. Sci.* **53**, 433, 1982.
3. Whitacre, C. C., Mattson, D. H., Day, E. D., Peterson, P. J., Paterson, P. Y., Roos, R. P., and Arnason, B. G. W., *Neurochem. Res.* **7**, 1209, 1982.
4. Wisniewski, H. M., and Keith, A. B., *Ann. Neurol.* **1**, 144, 1977.
5. McFarlin, D. E., Blank, S. E., and Kibler, R. F., *J. Immunol.* **113**, 712, 1974.
6. Pettinelli, C., and McFarlin, D. E., *J. Immunol.* **127**, 1420, 1981.
7. Ben-Nun, A., Wekerle, H., and Cohen, I. R., *Eur. J. Immunol.* **11**, 195, 1981.
8. Leibowitz, S., *In* "Multiple Sclerosis" (J. F. Hallpike, C. W. M. Adams, and W. W. Tourtellotte, Ed.), pp. 379–412. Williams & Wilkins, Baltimore, 1983.
9. Lisak, R. P., *Neurology* **30**, 99, 1980.
10. Hughes, R. A. C., Gray, A. I., Clifford-Jones, R., and Stern, M. A., *Acta Neurol. Scand.* **60**, 65, 1979.
11. Brinkman, C. J. J., Nillesen, W. M., Hommes, O. R., Lamers, K. J. B., dePauw, B. E. J., and Delmotte, P., *Ann. Neurol.* **11**, 450, 1981.
12. Newman, W., Stoner, G. L., and Bloom, B. R., *Nature (London)* **269**, 151, 1977.
13. Hensen, E. J., and Elferink, B. G., *Nature (London)* **277**, 223, 1979.
14. Morgan, D. A., Ruscetti, F. W., and Gallo, R. C., *Science* **193**, 1007, 1976.
15. Sredni, B., *J. Immunol. Methods* **49**, R1, 1982.
16. Sredni, B., Volkman, D., Schwartz, R. H., and Fauci, A. S., *Proc. Natl. Acad. Sci. USA* **78**, 1858, 1981.
17. Lamb, J. R., Eckels, D. D., Lake, P., Johnson, A. H., Hartzman, R. J., and Woody, J. N., *J. Immunol.* **128**, 233, 1982.
18. Boyum, A., *Scand. J. Clin. Lab. Invest.* **21**(Suppl 97), 77, 1968.
19. Kumagai, K., Itoh, K., Hinuma, S., and Tada, M., *J. Immunol. Methods* **29**, 17, 1979.
20. Breard, J., Reinherz, E. L., Kung, P. C., Goldstein, G., and Schlossman, S. F., *J. Immunol.* **124**, 1943, 1980.
21. Burns, J., Rosenzweig, A., Zweiman, B., and Lisak, R. P., *Cell. Immunol.* **77**, 363, 1983.
22. Deibler, G. E., Martenson, R. E., and Kies, M. W., *Prep. Biochem.* **2**, 139, 1972.
23. Kung, P., Goldstein, G., Reinherz, E. L., and Schlossman, S. F., *Science* **206**, 347, 1979.
24. Hashim, G. A., *Immunol. Rev.* **39**, 60, 1978.
25. Swanborg, R., *J. Immunol.* **130**, 1503, 1983.
26. Richert, J. R., Kies, M. W., and Alvord, E. C., *J. Neuroimmunol.* **1**, 195, 1981.
27. Ben-Nun, A., and Cohen, I. R., *J. Immunol.* **129**, 303, 1982.
28. Traugott, U., Reinherz, E. L., and Raine, C. S., *Science* **219**, 308, 1983.
29. Moller, G., "Models of Autoimmune Diseases," *Immunol. Rev.* **55**, 1981.
30. Arnason, B. G. W., and Antel, J., *Ann. Immunol. (Paris)* **129C**, 159, 1978.
31. Reinherz, E. L., Weiner, H. L., Hauser, S. L., Cohen, J. A., Distaso, J. A., and Schlossman, S. F., *N. Engl. J. Med.* **303**, 125, 1980.
32. Swierkosz, J. E., and Swanborg, R. H., *J. Immunol.* **119**, 1501, 1977.
33. Ben-Nun, A., Wekerle, H., and Cohen, I. R., *Nature (London)* **292**, 60, 1981.
34. Ben-Nun, A., and Cohen, I. R., *J. Immunol.* **128**, 1450, 1982.
35. Lamb, J., and Feldman, M., *Nature (London)* **300**, 456, 1982.

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SHORT COMMUNICATION

Differential Reactivity of Human Lymphocytes Allosensitized *in Vitro* in Hormonally Defined Medium or Medium Supplemented with Plasma

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We compared the *in vitro* allogeneic response of human lymphocytes cultured in hormonally defined medium (HDM) with that of those cultured in plasma-containing medium (PCM). Differences occurred only in secondary MLR or PLT and in cell-mediated cytotoxicity in terms of specificity, which was either greater or modified when HDM was used.

INTRODUCTION

The advantage of hormonally defined medium is well established for the analysis of cell proliferation and differentiation and also for the purification of soluble factors involved in the regulation of the immune response (1–3). Our aim was to analyze the inducer and effector cells of the human allogeneic response and their specificity by comparing the results obtained in normal plasma-containing medium with those obtained in hormonally defined medium. Thus, under the above-mentioned conditions, we studied the capacity of human lymphocytes (a) to proliferate in primary and secondary *in vitro* assays, (b) to generate cytotoxic effector cells, and (c) to produce suppressor regulatory factors (4, 5).

MATERIALS AND METHODS

Donors. Blood from nonrelated individuals and from family members, all HLA-A, B, and DR typed, was used.

Media. The tissue culture medium used was always RPMI 1640 (GIBCO, Grand Island, N.Y.) buffered with hydrogen carbonate and supplemented with antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, and 210 μg/ml fungizone) and 10% human pooled plasma. This medium was designated "plasma-containing medium" or PCM. Preparation of the hormonally defined medium or HDM is detailed elsewhere (1). It is composed of Iscove's modified Dulbecco medium (IMDM, GIBCO) with sodium bicarbonate (but no thioglycerol or β-mercaptoethanol), 1 μg/ml insulin, 0.5 μg/ml transferrin, and 1 mg fatty acid-free serum albumin reconstituted with 5

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μg/ml oleic acid, 5 μg/ml linoleic acid, and 1 μg/ml palmitic acid. Water was purified with a Pasteur pyrodistillator (Pasteur Institute Production).

Mixed-lymphocyte culture or MLR. Fresh or thawed peripheral blood lymphocytes were purified from heparinized blood on a Ficoll–Hypaque gradient (Pharmacia Fine Chemicals, Division of Pharmacia Inc., Piscataway, N.J.) (d = 1078 g/cm^3) and cultured in a V-bottom Microtiter plastic culture tray (Greiner, Nürtinger, West Germany) as described elsewhere (3).

Secondary cultures or PLT. Lymphocytes were sensitized *in vitro* by culturing 10 × 10^7 responder cells and 10 × 10^7 irradiated (2000 rad) stimulating cells in 20 ml PCM or HDM in plastic culture vials (Falcon 3013). The vials were incubated in an upright position at 37°C in a humidified 5% CO_2 air atmosphere. After 10 days incubation, the cells were harvested and stored in liquid nitrogen until secondary cultures had been performed. Sensitized responders were reconstituted from liquid nitrogen storage and adjusted to 0.5 × 10^6 viable cells/ml in either PCM or HDM. They were then restimulated with fresh or thawed X-irradiated stimulating cells. The cells were distributed into V-bottom Microtiter plastic culture trays (Greiner): 5 × 10^4 responders in 0.1 ml and 5.10^4 stimulators in 0.05 ml were added to each well. After 48 hr incubation, cultures were pulse-labeled for 6 hr using ^{3}[H]thymidine (2 μCi/well) (sp act: 1 Ci/mmol) and counted in a liquid scintillation counter (Intertechnic SL30).

Cell-mediated lympholysis or CML. Cells were allosensitized for 6 days in either PCM or HDM and used as effectors against phytohemagglutinin-treated ^{51}Cr-labeled target cells. Usually 10 × 10^6 target cells were labeled with 250 μCi of ^{51}Cr in 10 ml medium for 18 hr. The cytotoxic assay was performed in round-bottom microtiter plates (Linbro Chemical Co., Hamden, Conn.) with 5 × 10^5 effectors and 1 × 10^4 targets per well (at a ratio of 50:1). Microplates were incubated at 37°C for 4 hr and then centrifuged for 10 min. The supernatants were collected with a semiautomatic apparatus (Skatron, Titertek, Flow Laboratories Inc., Rockville, Md.) and counted in an Intertechnic γ scintillation counter. Spontaneous ^{51}Cr release was determined by target cells incubated in medium alone, and maximum release was determined by targets treated with 1 N HCl. Specific release (SR) was calculated with the formula:

$$SR = \frac{\text{counts per min (experimental)} - \text{counts per min (spontaneous)}}{\text{counts per min (maximal)} - \text{counts per min (spontaneous)}}. \quad [1]$$

Evaluation of suppression using suppressor supernatants. Suppressor supernatants, designated SF, were produced as follows: 10^7 lymphocytes, primed for 10 days *in vitro* and suspended in 10 ml PCM or HDM were irradiated at 2500 rad and cocultured again with 10^7 irradiated (6000 rad) initial stimulating cells. Supernatants were collected after 72 hr and stored at 4°C for brief periods or frozen at −80°C. The control supernatants, designated CF, were produced by unprimed lymphocytes from the same donors, incubated under the same conditions with autologous irradiated cells. Suppression was evaluated in preincubation experiments: 1 × 10^6 unprimed lymphocytes were incubated with 1 ml of SF or CF at 37°C in a CO_2 incubator for 15, 30, and 60 min. After being washed and centrifuged for 10 min at 600g, the preincubated cells were recovered and suspended in either PCM or HDM at a final concentration of 1 × 10^6 cells/ml. These cells were mixed in a primary proliferative assay, adapted from the classic MLR microtechnique, using 5 × 10^4 irradiated stimulating cells in 0.05 ml medium.

$$\% \text{ Suppression} = 1 - \frac{\text{cpm median value with cells preincubated with suppressor supernatant}}{\text{cpm median value with cells preincubated with control supernatant}} \quad [2]$$

RESULTS

Primary MLR. We performed a classic primary MLR with the cells of two unrelated individuals. These were incubated in either PCM or HDM. As shown in Table 1, the intensity of the proliferative response was increased and the peak response was delayed in HDM. We used a family genotyped for class I and II MHC antigens with two pairs of HLA-identical siblings and two nonrelated individuals who acted as controls. The results, presented in Table 2, were similar in both media; there was no allogeneic proliferation with HLA-identical pairs, and positive MLR were obtained in all other combinations within the family.

Secondary MLR. The lymphocytes of donor ING were sensitized for 10 days with the HLA-DR-different lymphocytes of donor LAM. These 10-day primed cells were then used in a 48-hr secondary MLR or PLT with four stimulator cells which had one or two HLA-DR antigens in common with the primary immunizing cell (LAM), and three stimulator cells HLA-DR different from the specific ones. The results obtained are expressed in Table 3. When PCM was used, proliferation was obtained only with the specific restimulating cells; no proliferation was observed with the nonspecific restimulating cells. On the other hand, when HDM was used, proliferation was observed not only with specific but also with nonspecific restimulating cells.

Cell-mediated lympholysis. We generated cytotoxic effector cells by mixing cells from two individuals in a primary MLR. The 6-day primed cells were harvested and tested against the specific primary stimulating cells and nonspecific target cells. With PCM, a positive CML was only obtained with specific targets (LAM) (Table 4), whereas the cytotoxic effector cells generated in HDM were found to kill both the specific and nonspecific targets (Table 4).

MLR suppressor factors or SF. We previously showed that *in vitro* hyperimmunized lymphocytes were able to produce MLR soluble suppressor factors or SF (4, 5). In this present study, we performed similar experiments using HDM. As shown in Table 5, either responder or stimulator cells were incubated with SF produced in PCM or HDM before being cocultured in MLR with HDM or PCM. As expected, when PCM

TABLE 1

Primary Mixed-Lymphocyte Reaction Using Two Unrelated Individuals

	Day 6	Day 7	Day 8
JAQ anti-JAQ	3[a]	3	3
	0.6[b]	3	3
JAQ anti-ANT	31	96	65
	52	34	18

[a] Median value obtained in HDM (cpm $\times 10^{-3}$).
[b] Median value obtained in PCM (cpm $\times 10^{-3}$).

TABLE 2

Mixed-Lymphocyte Reaction within Family PER

Responder cell	Stimulator cell	Father, JEA	Mother, GEN	Sibling BRI	Sibling OLI	Sibling BER	Sibling JAC	Unrelated individual, FAU
Father	ab[a]	2[b]	15	14	13	9	15	17
		0.9[c]	22	21	20	20	24	20
Mother	cd	9	1	9	13	13	13	16
		14	0.6	14	12	10	13	24
BRI	ad	10	12	2	1	9	9	11
		13	12	0.8	0.3	12	14	11
OLI	ad	7	20	1	2	10	9	14
		9	15	0.5	1	9	7	12
BER	bd	9	11	26	10	0.9	1	18
		14	12	19	13	0.4	0.8	24
JAC	bd	8	6	8	6	0.5	0.9	11
		9	9	9	12	0.4	0.3	14
FAU		12	20	27	10	11	10	0.5
		13	15	20	16	12	19	0.9

[a] HLA haplotype.
[b] Median value obtained in HDM (cpm × 10^{-3}).
[c] Median value obtained in PCM (cpm × 10^{-3}).

was used, an inhibition of proliferation occurred with both the preincubated responders and the preincubated stimulators. Similar results were obtained with HDM.

DISCUSSION

During the human allogeneic proliferative response *in vitro*, different subpopulations of lymphocytes are triggered and developed. We studied their function and specificity in plasma-containing medium and in hormonally defined medium. In the MLR performed with HDM, the modifications observed included an increased [^{3}H]thymidine incorporation and a delayed kinetic with a maximum response at Day 7 instead of

TABLE 3

Secondary Mixed-Lymphocyte Reaction Using Two Unrelated Individuals

	Restimulating cell[a]						
	HLA-DR specific				HLA-DR nonspecific		
	LAM HLA-DR7	MAU HLA-DR7,W6	CHI HLA-DR3,7	EVA HLA-DR4,7	ING HLA-DR2	REN HLA-DR1,W6	COU HLA-DR4
10-Day primed responder cells	35[b]	36	43	29	4	27	19
ING anti-LAM anti-HLA-DR7	16[c]	10	15	13	3	2	3

[a] Restimulating cells sharing or not sharing one HLA-DR antigen with the primary immunizing cells.
[b] Median value obtained in HDM (cpm × 10^3).
[c] Median value obtained in PCM (cpm × 10^3).

TABLE 4

Cell-Mediated Lympholysis Using Specific and Nonspecific Targets

Target	Effector:target cell ratio				
	40:1	20:1	10:1	5:1	2:1
Specific target, LAM	24[a]	30	20	11	0
	20[b]	22	13	12	9
Nonspecific target, DES	32[a]	32	28	21	6
	2[b]	9	0	0	0

[a] Percentage specific chromium release obtained in HDM.
[b] Percentage specific chromium release obtained in PCM.

Day 6 as observed with PCM. Moreover, the allorecognition was maintained as demonstrated by the family study in which all positive MLR combinations affected the HLA-different individuals, while no detectable proliferation could be seen in MLR performed with pairs of HLA-identical siblings. In contrast, differences were found when either PCM or HDM was used in secondary MLR or PLT. Using PCM, we obtained a positive secondary response with the 10-day primed responder cells *only* when the restimulating cells shared an HLA-DR antigen with the stimulator cells used in primary immunization of the responder cells (6, 7). When HDM was used, all secondary MLR were positive regardless of the stimulating cells used, suggesting that the specificity of the secondary MLR or PLT was lost or modified. However, loss of specificity is unlikely in view of the results obtained in primary MLR with the family studied. Similar data were obtained in cell-mediated cytotoxicity: effector cells produced in HDM were in fact able to kill both specific and nonspecific targets. On the other hand, no difference was seen in MLR preincubation experiments using suppressor factors produced in either PCM or HDM and tested in both media. The "wider" specificity of both the secondary proliferation and cell-mediated cytotoxicity in HDM, contrasting with the maintenance of the same suppressive activity in HDM or PCM, suggests that certain cell subsets could be preferentially developed in HDM. Conversely, the more "restricted" specificity of the reactions observed in PLT and CML, using PCM, could be explained by the presence of inhibitory factors in human pooled plasma. These inhibitors might prevent the recruitment of certain cell subsets. Moreover, HDM contains all the components present in normal tissue culture medium,

TABLE 5

Effect of Preincubation of Responders or Stimulators with SF from Donor DUP

	Responder[a]		Stimulator[a]	
Autologous producer, DUP HLA-DR1	20[b]	25[c]	24	29
Allogeneic sensitive responder, MAR HLA-DR6,4	34	31	20	23

[a] Responder or stimulator cells were incubated with SF from donor DUP for 30 min before being cocultured in MLR. Four stimulators were used for each responder in four different MLR.
[b] Mean percentage of suppression obtained with SF produced in PCM.
[c] Mean percentage of suppression obtained with SF produced in HDM, standard error never exceeded 10%.

but at different concentrations. This latter point raises the question of whether they could, for this reason, be interfering at cell membrane level; antigenic modification could occur and could be responsible for specific reactivity of allosensitized cells in HDM. It has been reported that the inhibition of certain cell subsets in PCM could be explained by a down regulation of specific membrane receptors. Wolfe *et al.* (8) have shown that the epidermal growth factor (EGF) is down-regulated by EGF in PCM. However, these specific membrane receptors remain on the cell surface when EGF is added in HDM.

Taken together, these preliminary data would seem to indicate that HDM might favor the recruitment of specific cell subsets, namely those involved in secondary MLR and cell-mediated cytotoxicity. Further investigations will be necessary to define their specificity more extensively and to elucidate the mechanisms responsible for the differential reactivity of allosensitized cells in PCM and HDM.

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REFERENCES

1. Hatzfeld, J., Hatzfeld, A., Maigné, J., Sasportes, M., Willis, R., and McClure, D. B., *In* "Cold Spring Harbor Conferences on Cell Proliferation," Vol. 9 "Growth of Cells in Hormonally-defined Media," pp. 703–710. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982.
2. Hatzfeld, J., Hatzfeld, A., and Maigné, J., *Proc. Natl. Acad. Sci. USA* **79**, 6280, 1982.
3. Barnes, D., and Sato, G. H., *Cell* **22**, 649, 1980.
4. Sasportes, M., Wollman, E. E., Cohen, D., Carosella, E., Bensussan, A., Fradelizi, D., and Dausset, J., *J. Exp. Med.* **152**, 270, 1980.
5. Sasportes, M., Carosella, E., Bensussan, A., Mihaesco, C., and Dausset, J., *Immunogenetics* **14**, 117, 1981.
6. Sasportes, M., Fradelizi, D., and Dausset, J., *Nature (London)* **276**, 502, 1978.
7. Sasportes, M., Fradelizi, D., Nunez-Roldan, A., Wollman, E., Giannopoulos, Z., and Dausset, J., *Immunogenetics* **6**, 29, 1978.
8. Wolfe, R. A., Wu, R., and Sato, G. H., *Proc. Natl. Acad. Sci. USA* **77**, 2735, 1980.